International Journal of Bio-Technology and Research (IJBTR)
ISSN (P):2249-6858; ISSN (E):2249-796X
Vol. 8, Issue 3, Jun 2018, 11- 22

Original Article

EFFECT OF PRIMING WITH GIBBERELLIN ON GERMINATION AND EXPRESSION OF GA BIOSYNTHESIS GENE OF TOMATO (SOLANUM LYCOPERSICUM L.) SEEDLINGS UNDER SALT STRESS

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ABSTRACT

Seed germination is the initial step of plant development. Seed priming with salt enhances seed germination in tomato (Solanum lycopersicum L.); however, the molecular and physiological mechanisms underlying the enhancement of seed germination by priming remain to be elucidated. Tomato seeds were soaked for 24 hrs at 25 ºC in the dark in different concentrations of NaCl (NaCl-priming) or distilled water (hydro-priming) and different concentrations of gibberellin. In this study, we examined the expression of genes encoding gibberellic acid (GA) biosynthesis enzymes, including GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox) and GA4 (GA 3beta-hydroxylase). The germination percentage and germination rate of tomato seeds were increased after soaking with GA under NaCl stress. The expression profiles of GA biosynthesis genes were investigated with the semi-quantitative RT-PCR. Additionally, the expression levels of GA biosynthetic gene were higher in the NaCl-primed seeds than in the hydro-primed seeds. These results suggest that the observed effect of NaCl-priming on tomato seed germination is caused by an increase of the GA content via GA biosynthetic gene activation that helps tomato plant to tolerate salt stress.

KEYWORDS: Seed Priming, Salt, Gibberellin, GA Biosynthesis Genes & Tomato

Received: May 09, 2018; **Accepted:** May 30, 2018; **Published**: Jun 20, 2018; **Paper Id.:** IJBTRJUN20182

INTRODUCTION

To improve seed-germination rates and field emergence under adverse environmental conditions, the pretreatment of seeds, termed seed priming, has been used in crop production. Appropriate priming treatments synchronies germination and improve seed performance in many crop species (Bradford et al., 1986 and Heydekker etal., 1973). By initiating germination processes before sowing, seed priming generally enhances germination and field emergence under adverse germination conditions (McDonald, 2000). Furthermore, pre-sowing seed treatment with an abiotic stress, such as sodium chloride (NaCl), enhances the tolerance of plants to saline (Strogonov, 1964). In tomato, priming treatments using KNO3, polyethylene glycol or NaCl have improved the germination, seedling emergence and the initial growth of plants (Alvarado et.al., 1987). According to a previous report, NaCl priming generally requires long-term treatment periods using solutions with relatively high concentrations of NaCl (Cano et al., 1991); however, short-term seed priming with a low NaCl concentration also increases germination rates, field emergence and acquired stress tolerance (Nakaune et al.,

2012). The effect of priming on transcription at early stages of seed germination has been investigated in several plant species, including Arabidopsis, *Brassica oleracea* and tomato (Bassel et al., 2008, Soeda et al., 2005 and Auge et al., 2009). Furthermore, the antagonistic roles of the plant hormones abscisic acid (ABA) and gibberellin (GA) in regulating seed germination are known (Karssen et al., 1989, Bewley, 1997 and Yamaguchi et al., 1998).

Presoaking seeds with optimal concentration of phyto hormones has been shown to be beneficial to growth and yield of some crop species growth under saline conditions by increasing nutrient reserves through increased physiological activities and root proliferation (Singh and Dara, 1971). Concerted attempts have been made to mitigate the harmful effects of salinity by application of plant growth regulators (Datta et al., 1998). Kabar (1987) suggested that endogenous hormone level is affected by many environmental stress however, external application of appropriate growth regulators optimize physical metabolic conditions for germination. It is also possible that under high salt concentrations naturally present hormones may be suppressed and that seed soaking with plant growth regulators supplies sufficient hormones for normal growth. Thus the detrimental effects of high salts on the early growth of wheat seedlings may be reduced to some extent by treating seeds with the proper concentration of a suitable hormone (Darra et al., 1973).

The molecular and physiological mechanisms underlying the effect of seed priming on germination in relation to plant hormones, however, are not fully understood. GA is important for seed germination, as evidenced by the inability of GA-deficient tomato (gib-1) and Arabidopsis (ga1-3) mutants to germinate without exogenous GA (Groot and Karrssen 1987 and Koornneef and van der Veen 1980). In Arabidopsis and other higher plants, genes encoding GA metabolic enzymes have been isolated, including copalyl diphosphate synthase (CPS), ent-kaurene synthase (KS), ent-kaurene oxidase (KO), ent-kaurenoic acid oxidase (KOA), GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox), GA 2-oxidase (GA2ox) and GA4(GA 3beta-hydroxylase)(Hedden and Kamiya 1997 and Hedden and Phillips 2000). The expression levels of these genes have been analysed during the germination process, as have endogenous GA concentrations (Ogawa et al., 2003), and several reports have shown that their expression is regulated by both endogenous and environmental conditions (i.e., light and temperature) (Yamaguchi and Kamiya, 2000, García-Martínez and Gil, 2002, Yamauchi., et al., 2004 and Seo et al., 2006). In tomato, the GA biosynthetic genes CPS, GA20ox, GA3ox, GA2ox and GA4 have been isolated (Rebers et al., 1999 and Serrani et al., 2007). The process of fruit set in tomato involves GA1 as the bioactive GA molecule and the early 13-hydroxylation pathway as the primary metabolic pathway (Serrani et al., 2007 and Fos et al., 2000). Pollination mediates fruit set through GA biosynthesis, which is mainly achieved by the up-regulation of GA20ox (Serrani at al., 2007). For seed germination in tomato, however, the relevant GA biosynthetic processes and changes in GA levels remain unclear. To obtain a better understanding of the enhancement of tomato seed germination by NaCl priming, in this paper, we investigated the germination percentage and germination rate of tomato seeds after soaking with GA under NaCl stress and the expression profiles of GA biosynthesis genes with the semi-quantitative RT-PCR. The results indicate that NaCl-priming enhances transcription of the gene for GA biosynthesis.

MATERIAL AND METHODS

Plant Materials and Priming Treatments

Tomato seeds (*Solanum lycopersicum* L.) Castlerock VF variety from Pacific Seed Company were used for all experiments. The seeds were soaked for 24 hrs at 25 ºC in the dark in 1000, 3000, 4000 mM NaCl (NaCl-priming), distilled water (hydro-priming) and 2 concentrations of GA (300,600 ppm) (GA-priming). After the priming treatment, the seeds were washed with distilled water. A piece of filter paper was placed into each of 100 mm × 15 mm Petri dishes, and 5 ml of a test solution was added. The seeds were transferred onto the filter paper, with 30 seeds per dish and a 3 mm or larger distance between each seed. The combinations of GA concentrations and the NaCl were added to Petri dishes along with 5 ml of each solution. Pure water used for the control treatment. The Petri dishes were covered, sealed with tape and incubated at room temperature. Seed germination was defined as the protrusion of the radicle through the seed coat. The germinating seeds were counted based on 2 mm of radical emergence 24 hrs after planting. The counting continued until the number of germinated seeds was constant for at least three days. The germination counting was halted after 8 days. From each dish, 10 seedlings were selected randomly. The seed germination rate and the mean germination time were calculated.

Seed Germination Measurement

The final germination percentage was calculated based on the total number of germinated seeds at the end of the experiment. The measurements were conducted according to the International Rules for Seed Testing (ISTA, 1996). The germination indices were calculated using the following equations (Ellis and Roberts, 1981).

Germination percentage (GP%) = (Gf/n) × 100(1)

Where; Gf = the total number of germinated seeds at the end of experiment, and n = the total number of seeds used in the test.

Mean germination time (MGT) = Σ NiDi/n(2)

Where; Ni = the number of germinated seeds until the ith day, Di = the number of days from the start of experiment until the ith counting, and n = the total number of germinated seeds.

Germination rate (GR) = Σ Ni/ΣTiNi(3)

Where; Ni = the number of newly germinated seeds at the time of Ti. = (a/1)+(b-a/2)+(c-b/3)+...+(n-n-1/N)

Semi-Quantitative RT-PCR Analysis

The expression of GA biosynthesis genes was examined using semi-quantitative RT-PCR. Many genes from tomato have been identified and recorded in previous studies that were involved in GA biosynthesis. In this study, tomato GA biosynthesis genes were selected based on the availability of their ESTs sequence in SGN database (http://solgenomics.net/), a tomato genome database (SGN, 2017). These sequences were used to design the gene-specific primers. The total RNA was extracted from tomato seedlings with TRIzol reagent (Sigma, USA), which was then reverse-transcribed into cDNA using the RT-PCR Superscript™ II reverse-transcriptase kit (Invitrogen, USA) with the Oligo(dT) primers. The EST sequences available in the SGN database were used to design gene-specific primers using Primer3 software (version v. 0.4.0) (http://frodo.wi.mit.edu/primer3/). The PCR was used to amplify the cDNAs for each of the

investigated tomato genes using combinations of forward and reverse primers with the synthesized cDNA as a template. 5 templates were tested for the expression analysis of each GA biosynthesis gene, which included the following treatment combinations: the NaCl1000GA300 and NaCl4000GA600 treatments and their controls, the NaCl1000 and NaCl4000 alone treatment and the non-treated control. The PCR reaction volume was 20 µl that contained 4 µl of 5X PCR ready mix that contained Taq polymerase (Solis BioDyne, Estonia), 0.4 µM of each primer, 3.5 µl of cDNA and sterile distilled water to a final volume of 20 µl. The amplifications were performed under the following conditions: an initial denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at a 60ºC(see Table 1), and an extension at 72°C for 1.5 min. An extension at 72°C for 7 min was performed after all cycles were completed. The constitutive expressed gene, clathrin adapter complexes (CAC), was used as positive control. The PCR products were separated on 1.2% agarose gel (Sigma, USA). The electrophoresis was then performed for 30 min at 100 volts in Tris/borate ethylenedia- minetetraacetic acid (EDTA) buffer (TBE) (Sigma, USA). The products were stained with 3 µl of 10 mg/ml ethidium bromide (Sigma, USA) in 120 ml of TBA buffer. The stained bands in the gel were visualized and documented using a gel documentation system (Bio-Rad Laboratories, USA) with a UV light and Gel Doc 2000 Quantity One software. Each gene expression analysis was performed at least three times as independent PCR reactions and electrophoresis on gels, and one of the images is presented as a representative for each gene.

Table 1: Primer Combinations used for Semi - Quantitative RT-PCR Analysis 3 Tomato GA Biosynthesis Genes, in Addition to the Internal Control Gene, CAC were used. Forward and Reverse Primers Designed from Tomato Salt Stress Genes Sequences Available on of SGN Database` Http://Solgenomics.Net/

Gene	Accession #	Forward and Reverse Primers	Annealing temperature	Product Size (bp)
GA3OX1	XM_004229049.2	5'-GTGGAACTTATGGGCCATGC-3' 5' CGCCTTGCCTGAGATTCAAC-3'	60 ºC	104
GA20OX 1	EU043161.1	5'-AGATGACACCAGCACTGACT-3' 5'-GAGTTTCCTTTGGTTGGGTGA-3'	60 ºC	106
GA4	NM_001246919.2	5'-ACGTACCACACTATGCCTTCA-3' 5'-CACCCCATTTCTTGCATGCA-3'	60 ºC	250
CAC	08g006960.2.1	5'-CCTCCGTTGTGATGTAACTGG-3' 5'-ATTGGTGGAAAGTAACATCA-3'	60 ºC	173

RESULTS

Germination of Nacl and GA Primed Seeds

In the current research, we used a germination test to confirm the effects of seed priming and investigate the role of GA in enhancing tolerance of tomatoes to salinity and highlighted the expression profiles of GA biosynthesis genes in tomato plants at germination stage. Three priming groups were used in germination experiments with 3 replicates of each treatment. For the first group, the plants were grown in distilled water only which was considered as hydro-priming. For the second group, the plants were grown in 1000, 2000, 3000 and 4000 mM NaCl without GA, which was considered as NaCl priming. For the third group, the plants were grown in the same concentrations of NaCl with 300 and 600 ppm GA respectively, which was considered as GA priming. The mean germination time required for the initiation of germination and was not different among the NaCl-, hydro- and GA-primed seeds; however, the GR and GP of the NaCl- and GA-primed seeds were significantly higher than hydro-primed seeds (Table 2, Figure 1). The GR and GP of the GA-primed seeds at 300 ppm (Table 3, Figure 2) were better than at 600 ppm (Table 4, Figure. 3). Our results indicated

that GA with optimum concentration played an important role in moderating the inhibition of seed germination and plant growth in saline environments with the induction of salt tolerance in tomato plants.

Table 2: Influence of Different Concentrations of Nacl on Tomato Germination without GA

No. of replicates		0	1000	2000	3000	4000
1	GP%	93.33	86.67	86.67	83.33	83.33
2	GP%	83.33	93.33	90.00	93.33	83.33
3	GP%	90.00	83.33	96.67	83.33	83.33
1	GR	7.70	7.67	6.62	5.67	5.75
2	GR	7.00	7.90	6.88	6.82	5.65
3	GR	7.57	6.87	7.62	5.75	5.58
1	MGT	24.57	23.93	22.13	20.97	20.07
2	MGT	23.23	23.73	24.40	21.53	20.07
3	MGT	24.03	22.33	25.10	20.33	19.73

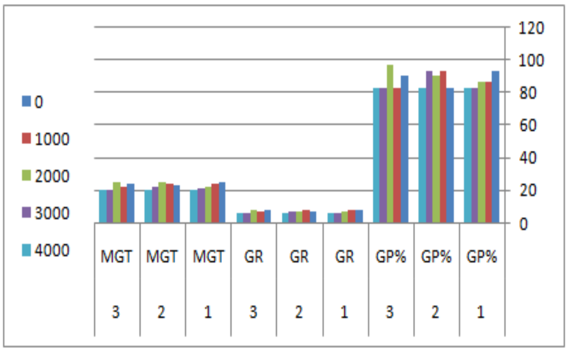

Figure 1: Chart Represents the Influence of the Different Concentrations of Nacl on Germination Efficiency and Seedling Growth of Tomato Plants without GA. Horizontal Axis in All Charts Present Nacl Concentration (0, 1000, 2000, 3000 And 4000 Mm) as Salt Stress Vertical Axis Presents: Germination Percentage (GP), Germination Rate (GR) and Mean Germination Time (MGT)

Table 3: Influence of different concentrations of NaCl on tomato germination with GA at (300 ppm)

No. of replicates		0	1000	2000	3000	4000
1	GP%	86.67	96.67	86.67	93.33	83.33
2	GP%	93.33	83.33	93.33	93.33	86.67
3	GP%	96.67	86.67	83.33	90.00	90.00
1	GR	7.70	8.67	6.80	6.88	6.40
2	GR	8.83	7.42	7.40	7.45	6.90
3	GR	9.08	7.92	7.20	7.02	6.20
1	MGT	24.23	26.30	23.00	24.47	21.83
2	MGT	27.00	23.60	23.07	23.80	22.20
3	MGT	27.33	24.23	22.73	23.30	21.93

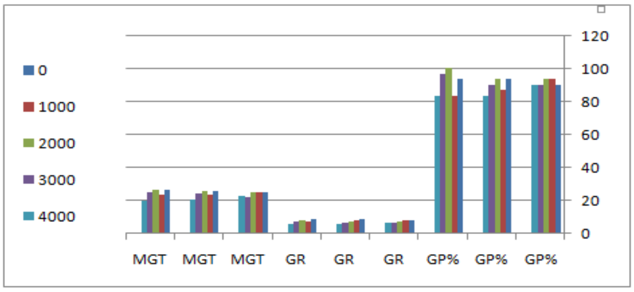

Figure 2: Chart Represents the Influence of the Different Concentrations of Nacl on Germination Efficiency and Seedling Growth of Tomato Plants with GA at 300 Ppm. Horizontal Axis in All Charts Present Nacl Concentration (0, 1000, 2000, 3000 And 4000 Mm) as Salt Stress Vertical Axis Presents: Germination Percentage (GP), Germination Rate (GR) and Mean Germination Time (MGT)

Table 4: Influence of Different Concentrations of Nacl on Tomato Germination with GA At (600 Ppm)

No. of replicates		0	1000	2000	3000	4000
1	GP%	90.00	93.33	93.33	90.00	90.00
2	GP%	93.33	86.67	93.33	90.00	83.33
3	GP%	93.33	83.33	100.00	96.67	83.33
1	GR	7.77	7.82	7.45	6.37	6.45
2	GR	8.50	7.95	7.65	6.43	6.18
3	GR	8.58	7.58	8.45	7.63	5.82
1	MGT	24.80	24.90	24.90	22.10	22.60
2	MGT	26.00	23.80	25.97	24.03	20.57
3	MGT	26.17	23.27	26.87	25.30	20.23

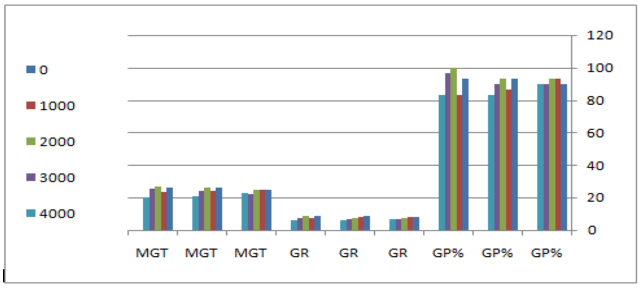

Figure 3: Chart Represents the Influence of the Different Concentrations of Nacl on Germination Efficiency and Seedling Growth of Tomato Plants with GA at 600 Ppm. Horizontal Axis in all Charts Present Nacl Concentration (0, 1000, 2000, 3000 And 4000 Mm) as Salt Stress. Vertical Axis Presents: Germination Percentage (GP), Germination Rate (GR) and Mean Germination Time (MGT)

Expression Profiles of GA Biosynthesis Genes in the Nacl-Primed Seedlings

The expression of the GA biosynthesis genes was investigated with semi-quantitative RT-PCR using the cDNA isolated from tomato seedling tissues. Two of the treatment combinations, NaCl1000 GA3000and NaCl2000GA400, with their respective controls, NaCl1000 and NaCl4000 GA0, in addition the non- treated control, were chosen for the semi-quantitative RT- PCR analyses. As shown in Figure 4. The mRNAs for the CAC gene, used as a positive control, were expressed approximately uniformly in all the tested treatments and their controls.

Compared with the hydro-primed seedling, the 3 GA biosynthesis genes (GA3OX1, GA20OX1 and GA4 were up-regulated by NaCl-priming (Figure. 4). For instance, the expression of the genes most likely related to the promotion of plant growth through auxin signaling. The expression levels of the GA biosynthesis genes were higher in GA 300 ppm than in GA 600 ppm (Figure. 4). This means that GA 300 ppm is considered as an optimum concentration for mitigating the effect of salt stress.

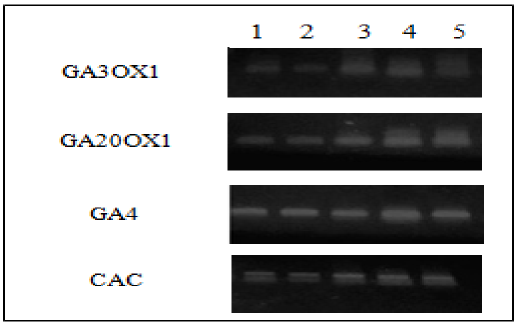

Figure 4: Semi-Quantitative RT-PCR Analysis of 3 GA Biosynthetic Genes Expressed in Tomato Seedlings under Two Concentrations of Nacl, Nacl1000 and Nacl4000, and Two GA Concentrations 300ppm and 600 Ppm in Addition to the Non-Treated Control Plants. the CAC Gene was used as a Positive Control. Lane 1: Nacl0 GA0, Lane 2: Nacl1000 GA0, Lane 3: Nacl4000 GA0, Lane 4: Nacl1000 GA 300, Lane 5: Nacl4000 GA600

DISCUSSIONS

Seed-priming techniques have been used to improve both seed germination and seedling emergence and have been adapted for tomato (Alvarado et al., 1987; Argerich and Bradford 1989; Cano et al., 1991; Cayuela et al., 1996). The eventual germination ratio in the present results was not so different from the previous reports using much higher salt concentrations. However, in this study, we demonstrated that NaCl-priming is effective enough to promote seed vigor as well as germination in tomato. Additionally, the 24 h of treatment duration which we adopted in this work is actually shorter compared to the 36 h–6 d of duration in conventional methods (Alvarado et al., 1987; Argerich and Bradford et al., 1989; Cano et al., 1991; Cayuela et al., 1996), indicating our method would contribute to save time as well as cost for priming.

One possible explanation for the early field emergence of the NaCl-primed seeds is the promotion of seed germination by NaCl treatment. The process of germination in tomato seeds has been studied in some details. The tomato embryo is surrounded by a rigid endosperm. When germination begins, as a first step, the endosperm that encloses the radicle tip, and the endosperm cap weakens to allow the radicle to emerge (Groot and Karrssen 1987). Enzymes, such as endo-β- mannanase, expansin, β-1,3-glucanase and xyloglucan endo trans glycosylase, are thought to be involved in the weakening of the endosperm cap. Gibberellin (GA) induces the transcription of those genes. The enzymes responsible for the weakening of the endosperm cap and a process related to germination are regulated by plant hormones, such as ABA and GA. To increase our understanding of the effect of NaCl-priming on germination, a further investigation of how the plant hormones function in the germinating seed to regulate the expression of genes encoding the enzymes required for endosperm cap weakening under priming treatments is needed. The potential improvement of seedling growth by seed priming is widely recognized. In our study, the germination parameters of seedlings primed withNaCl were higher than those of the non-primed or hydro-primed seedlings (Table 2, Figure 1). Our results are consistent with the previous reports, suggesting that the growth promotion results from improved seedling emergence and germination by NaCl-priming. Auxin

participates in many aspects of the developmental response in plants, including growth, cell expansion and differentiation (Friml 2003). The results of the GA biosynthesis gene expression profile indicated an up-regulation of the genes related to GA signaling, such as GA3OX1, GA20OX1, GA4 in the NaCl-primed seeds (Figure 4). It has also been reported that the auxin concentration is increased in the NaCl treated tomato root (Albacete et al., 2008), suggesting the increased auxin concentration in NaCl-primed seeds/seedlings and the subsequent up-regulation of auxin responsive genes. It has been reported that priming enables seeds to germinate more efficiently under unfavorable conditions, such as high or low temperatures (Rumpel and Szudyga 1978; Georghiou et al., 1982). However, it has been reported that in some tomato cultivars grown under saline conditions, the fruit yield was higher in plants from primed seeds than in plants from non-primed seeds. This effect was more remarkable when the salt treatment was applied during germination than when it was applied at the seedling stage (Cano et al., 1991). It has also been reported that plants exhibit a higher capacity to adapt to salinity when salt treatments were applied during germination than when it was applied after emergence (Bolarín et al., 1993). Similarly, our results showed that under NaCl-stress conditions, the germination rate of NaCl primed seeds was higher than that of the non-primed and hydro-primed seeds during the early germination stages (Table 2, Figure, 1). With regard to the gene expression profile, NaCl-priming induces expression of GA3OX1, GA20OX1, GA4 (Figure. 4). It has been reported that up-regulation of GA biosynthesis genes under salt stress might be related to protection against salt induced oxidative stress (Najami et al., 2008). Thus, it was strongly suggested that the higher germination ratio under the salinity stress in the NaCl- primed seed was caused by the modified gene expression and that this higher ratio would affect the subsequent adaptability to the salinity. It has been reported that plant hormones participate in this process by inducing the expression of genes associated with stress tolerance (AbuQamar et al., 2009; Amitai-Zeigerson et al., 1995). The relationships among seed priming, stress tolerance and plant hormones still remain unclear in tomato. Therefore, in addition to seed germination, it is important to examine the changes in the plant hormone levels in the primed plant.

CONCLUSIONS

Our results demonstrate that salt seed priming is effective in the promotion of germination and seedling emergence and to enhance the stress tolerance and subsequent growth of tomato plants. These processes are accompanied by modification of the expression of genes related to GA biosynthesis. This established method will contribute to the production of high-quality tomato seedlings. Before applying the method to other crops, it will be important to elucidate the biochemical and molecular mechanisms underlying this phenomenon.

ACKNOWLEDGEMENTS

The author is greatly indebted to Maha Al-Roais, research assistant in the Biology Department, College of Science and Humanities, Prince Sattam bin Abdulaziz University, for her excellent research assistance.

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